

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
15 March 2001 (15.03.2001)

PCT

(10) International Publication Number
WO 01/18161 A2

(51) International Patent Classification⁷: **C11C 3/00**

(21) International Application Number: **PCT/IB00/01274**

(22) International Filing Date:
8 September 2000 (08.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
MI99A001894 9 September 1999 (09.09.1999) IT

(71) Applicant and

(72) Inventor: **GHISALBERTI, Carlo** [BR/BR]; Rua Luis Dias 85/61, Itaim-Bibi, CEP-04542-080 São Paulo, SP (BR).

DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— Without international search report and to be republished upon receipt of that report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,

(54) Title: NEW PROCESS FOR THE PREPARATION OF CONJUGATED LINOLEIC ACID (CLA)

(57) Abstract: The present invention relates to a new solvent-free process the preparation conjugated linoleic acid (CLA). The present invention also relates to CLA obtainable by alkali isomerization of grape seed oil as new source of highly pure linoleic acid as well as to a synthetic procedure for the manufacture of CLA-triglyceride with a high content of 9c,11t-octadecadienoic acid.

WO 01/18161 A2



NEW PROCESS FOR THE PREPARATION OF CONJUGATED LINOLEIC ACID (CLA).

FIELD OF THE INVENTION

The present invention relates to a new process the preparation
5 conjugated linoleic acid (CLA).

More particularly, the present invention relates to a solvent-free synthetic procedure for the manufacture of CLA.

The present invention also relates to CLA obtainable by alkali isomerization of grape seed oil as new source of highly pure linoleic
10 acid as well as to a synthetic procedure for the manufacture of CLA-triglyceride with a high content of 9c,11t-octadecadienoic acid.

The CLA obtained by the procedures of the present invention are particularly suitable for human use by topical and oral administration.

15 BACKGROUND OF THE INVENTION

Conjugated linoleic acid (CLA) is a mixture of positional and configurational isomers of octadecadienoic acid, which are naturally occurring substances found in milk and diary products as well as in meats of ruminants.

20 The term CLA includes the family of positional and configurational isomers of C18:2 fatty acid, more precisely the cis and trans form of 9,11- 10,12- and 11,13-octadecadienoic acids.

Many studies reported that synthetic CLA is an effective agent in inhibiting mammary, colon, forestomach, and skin carcinogenesis in
25 experimental models, due to its modulation of lymphocyte and

macrophage activities. Recent clinical and in vivo data disclosed novel biological effects of CLA, e.g. the anti-atherogenic and anti-hyperinsulinemic activities.

CLA is a biologically active ingredient which may be suitably used as nutritional supplement in combination with food antioxidant, as disclosed and claimed in our copending application.

Although CLA naturally occurs in a wide variety of food, particularly in beef and dairy products, the normal food intake could not be adequate to provide the desired effects. This array of properties has drawn the focus of the fine chemical industry on the methods of manufacturing a CLA suitable for human consumption, thus with the necessary levels of purity and safety for the use as nutritional supplement.

Several prior proposals produced CLA admixtures in the presence of alkali bases. In US2350583 a method to produce CLA is described by heating a reaction mixture of an aqueous solution of alkali soaps of the fatty acids containing an excess of alkali in water for several hours at elevated temperature and under autogenic pressure.

A continuous flow process for conjugating unconjugated fatty acids is illustrated in US4164505, where the resulting fatty acid mixtures is poorly controllable, thus it is used as drying oil in application such as alkyd paints, lacquers and varnishes.

Similar isomerization processes operating under substantially anhydrous conditions are for example disclosed in US2389260 and US2242230.

Another prior proposal utilizes an ether of a polyhedric alcohol which contains a free hydroxyl group as solvent to dissolve the fatty acid soaps to performe the isomerization reaction.

CLA is also produced by the alkali isomerization of high linoleic
5 acid oils, typically carried out in high boiling point solvents such as ethylene glycol, as reported in JAOCS, 36, 631 (1959), where methyl linolenate was heated in a solution of KOH and ethylene glycol at 200°C for 7 hours to yield aproximately 80% CLA.

According to the 34th Annual Meeting on Oil Chemist's Society, p.
10 171 (1995), tests were carried out in accordance with the method described in the "Standard Methods for the Analysis of Fats, Oils and Related Materials", 2.4.16-17, when methyl linolate is reacted in a solution of KOH and ethylene glycol at 180°C for 2 hours, the conversion to CLA is about 80% with a six-fold excess of KOH, whereas
15 when safflower oil is isomerized with 2 equivalents sodium methoxide in solvents such as dimethyl sulfoxide and dimethylformamide, the conversion to CLA is approximately 73%.

A method for producing CLA, by submitting a linoleic acid containing fat or oil to an isomerization reaction in an alkali-propylene
20 glycol solution, is disclosed in EP0839897.

The use of an ether of a polyhedric alcohol which contains a free hydroxy group as a solvent to dissolve the fatty acid soaps during the isomerization is disclosed in US2343644, and similar isomerization processes operating under substantially anhydrous conditions are
25 disclosed in US2389260 and US2242230.

Among the various solvents reported in the above reference, the alkylene glycols are preferred from the viewpoint of alkali solubility, whilst dimethyl sulfoxide and dimethylformamide must be used in large amounts due to the low solubility of the alkali thereon.

5 However, all the aforementioned high boiling solvents have some degrees of toxicity. Moreover such high boiling solvents must be removed with low boiling organic solvents, such as heptane, in order to allow the phases separation during the alkaline neutralization to recover free CLA. Despite the organic solvent being removed by
10 vacuum stripping, residual solvent and highly toxic glycol ether may be present in the final product.

DETAILED DESCRIPTION OF THE INVENTION

It is appreciated that in the present specification the term CLA is intended to include either CLA in the form of free fatty acid or its
15 derivatives, such as its phospholipid, its mono-, di- and tri-glycerides, ethers, esters or salts thereof. All derivatives must be physiologically acceptable, i.e. non-toxic derivatives of CLA. Preferred salts of CLA include the metallic soaps of CLA with alkaline and/or earth-alkaline ions, such as sodium, potassium, or magnesium ions, and the nitrogen-
20 containing salts, such as ammonia, mono-, di- or tri-ethanolamine.

According to one of its aspects, the present invention relates to a new process for the preparation of CLA, overcoming the drawbacks of the known methods. CLA is thereby prepared by a new solvent-free process providing high purity CLA with almost no trace of further
25 contaminants and by-products.

Therefore, a first object of the present invention is a solvent-free process for the preparation of CLA, by alkaline isomerization of a vegetable oil in presence of an excess of free glycerol.

More particularly, the invention concerns a process for the
5 preparation of CLA which comprises reacting conjugated vegetable oils with an excess glycerol and an appropriate alkali metal hydroxide at high temperature, such as from about 200 °C to about 250°C, preferably around 230-235°C.

The term "vegetable oils" defines in the present specification a
10 natural vegetal oil as well as any high linoleic fatty acid products.

The present process performs efficiently on high linoleic fatty acid, e.g. distilled 90% linoleic acid methyl ester but good quality CLA may also be produced from other selected vegetal oil containing appreciable content of linoleic acid, for example from about 50% to
15 about 65% of linoleic acid.

Vegetable oils of the most practical interest are those with the highest linoleic acid content coupled with the lowest linolenic acid content, such as grape seed oil, safflower oil, and sunflower oil.

The process comprises reacting conjugated vegetable oils with
20 an excess glycerol and an appropriate alkali metal hydroxide at high temperature, such as from about 200 °C to about 250°C, preferably around 230-235°C.

The present process is carried out by charging the linoleic acid-containing oil with glycerol and alkali metal hydroxide into a reaction

vessel. Alternatively, the glycerol can be charged separately to the reaction vessel to a previously formed solid soap of the fatty acids.

Typically, glycerol is charged into the vessel in an adequate amount to dissolve the alkali metal soaps which formed therein at about 100 °C. Generally, from about 50% to about 200% glycerol by weight of the remaining components of the reaction mixture is used in the present process. Preferably, the glycerol to vegetable oil ratio is 1:1 w/w.

The reaction vessel, held under nitrogen atmosphere, is heated to a temperature of from about 200°C to 250°C, advantageously from about 210 °C to 235 °C, a temperature of about 235°C being preferred. The reaction temperature should not exceed the activation temperature of acroleine and glycerol ethers formation (above 255 °C).

The use of a high flow nitrogen stream should be avoided to minimize dehydrating conditions. Reaction times for the present process generally range from about 3 to about 6 hours, depending on a variety of factors such as the reaction temperature, the concentration of linoleic acid of the starting material, the desired composition of CLA, and the alkali concentration.

The process is virtually complete when the original linoleic acid content is less than 2%; at this point the reaction mixture is allowed to cool. During cooling the reaction mixture tends to build up viscosity, therefore the addition of water and heating for about 0,5 to 1 hour to complete saponification of any residual monoglyceride is particularly

recommended. In fact, if the saponification is incomplete, the residual amounts of monoglycerides may prevent the phase separation by formation of a cloudy emulsion.

The acidulated CLA forms a lower hydroglyceric layer and an
5 upper fatty acid oil layer containing CLA, which is then separated by decantation, centrifugation or the like. CLA may then be washed with water or with a steam flow. Preferably, CLA is then made anhydrous by sodium sulphate.

The alkaline agent is preferably a water soluble alkali metal base
10 such as an alkali metal hydroxide or oxide. The amount of alkali metal hydroxide to be used is selected in order to completely (stoichiometrically) saponify the whole fatty acid content of the high linoleic fatty acid or ester from selected vegetal oil sources, the neutralization point being reached at a pH of about 11.7. Preferably a
15 small excess of the stoichiometric amount is used so that a minor amount of free alkali is still present in the final glyceric reaction mixture. Typically about 1% to 50% of stoichiometric alkali excess provides a good working range for the present process, though the lowest excess alkali quantities is preferred.

20 According to a preferred embodiment, potassium hydroxide is preferred over sodium hydroxide since the CLA potassium soaps display a lower viscosity at 50-90°C than the corresponding sodium soaps, hence easing the recovery of the product.

In order to obtain a high grade CLA, the product can be
25 optionally further refined by conventional refining techniques,

including drying under vacuum, clarification by bleaching earth, stripping as vacuum distillation techniques or the like, to provide a purified, odourless and tasteless CLA.

The alkaline isomerization of linoleic acid produces a complex
5 mixture of positional and configurational isomers of CLA in form of free fatty acids or alkaline soaps thereof. For instance, the aforementioned process of the present invention provides a CLA containing a full range of CLA isomers in the 11,13, 9,11 and 10,12 positions, and the combination of cis and trans configuration. The c9,c11, c9,t11, and
10 furthermore c10,c12, t10,c12 isomers are the most abundant among CLA components, whereas a minor fraction of the t,t-isomers are generally formed by the further isomerization of c9,t11- or t10,c12-isomers.

This "mixed" CLA is particularly suitable for the oral
15 administration in combination with food grade antioxidants, as recently pointed out by Gavino VC, Gavino G, Leblanc MJ, Tuchweber B in "An isomeric mixture of conjugated linoleic acids but not pure cis-9,trans-11-octadecadienoic acid affects body weight gain and plasma lipids in hamsters." J. Nutr. 130:27-29
20 (2000).

A particularly preferred vegetal oil for performing the process of the present invention is grape seed oil.

The use of grape seed oil for the production of CLA is new and represents another aspect of the present invention.

Also, another object of the present invention is CLA obtained by alkali isomerization of grape seed oil in solvents having boiling point higher than 240°C.

Grape seed oil contains the average largest amount of linoleic acid, namely 72-76% by weight - therefore higher than sunflower oil (70-72%), sunflower (60-62%), and corn oil (about 52%) – coupled with the minimum level of linolenic acid, whose isomerization ends up in highly unsaturated conjugated acids of hazardous metabolic impact.

Moreover, we have found out that the CLA obtained by grape seed oil, regardless the isomerization procedure applied, is more resistant to peroxidation. This is probably due to the residual presence of antioxidant polyphenols naturally occurring in grape seed, i.e. the oligomeric proanthocyanosides, which shall account for the increased oxidation resistance of the end-product. Said CLA is particularly suitable for both topical and oral uses.

For some pharmaceutical, nutritional or cosmetic applications the CLA may be more suitable in its triglyceride form and/or with an high content of the most abundant of the positional and configurational isomers, i.e. 9c,11t- and 9,t-11t-octadecadienoates.

The CLA-triglyceride may a be obtained from CLA-free fatty acids by known transterification methods. For example, CLA fatty acids, either from dehydrated ricinoleate or from vegetable oil isomerization, may be converted in the corresponding methyl esters

and then reacted with triacetine (triacetyl-glycerine) and sodium ethoxylate under reduced pressure to provide CLA-triglyceride.

Highly pure c-9,t-11-CLA may be conveniently obtained by chemical dehydration of ricinoleic acid and esters by known methods. An example is illustrated by Berdeaux O, Christie WW, Gunstone FD, Sebedio JL. in "Large-scale synthesis of methyl cis-9,trans-11-octadecadienoate from methyl ricinoleate". J. Am. Oil Chem. Soc. 74:1011-15 (1997).

We have now found a direct method of producing a CLA-triglyceride with a high content of 9,11-CLA isomers from castor oil.

Therefore, according to another of its aspects, the present invention relates to a process for the preparation of CLA-triglyceride from castor oil by binding a leaving group in the 12-position of the starting castor oil, reacting said castor oil with mineral acids and cleaving of said leaving group with a suitable tertiary amine.

The term "customary leaving group" defines in the present specification a group which is more appropriate for cleavage than the hydroxy group.

The first step can be carried out by reaction of castor oil with tosyl or mesyl chloride in presence of a tertiary amine, such as pyridine, triethylamine or dimethylaminopyridine (DMAP) in presence of suitable organic solvents. The resulting tosylated or mesylated castor oil may be then treated with diluted mineral acid, such as HCl or sulphuric acid to allow phase separation. The organic phase is repeatedly washed with acidulated water, dried over sodium

sulphate, then reacted with a further tertiary amines at high temperature to yield CLA in its triglyceride form.

Examples of suitable tertiary amines are diazabicyclo-undecene (DBU), diazabicyclo-nonene (DBN) and DMAP.

5 After the reaction, the mixture is added with water and an organic solvent, e.g. heptane, repeatedly washed with dilute acids and then dried.

The following examples show in detail how the present invention can be practiced but should not be intended as limiting it.

10 Example 1 - Synthesis of CLA by alkaline isomerization of grape seed oil in glycerol

1 kg glycerol, 235 g potassium hydroxide (KOH) and 1000 g of grape seed oil were added into a 4-neck round bottom flask (5000 ml) equipped with a mechanical stirrer, a thermometer, a reflux
15 condenser, and a nitrogen inlet, the nitrogen being introduced in first run through two oxygen traps.

Nitrogen was bubbled into the reaction mixture for 20 min and the temperature was then raised to 90-100 °C, and kept under mechanical stirring for about 20 minutes to convert the triglyceride in
20 the corresponding potassium salts. The double phase system disappears to form a glyceric soap suspension, then heated at 230 °C under inert atmosphere and stirred for 4 hours.

The reaction mixture was cooled to about 100 °C, and the stirring stopped as the reaction mixture tend to reach very high
25 viscosity during cooling. 2 l of water was then slowly added, and the

mixture kept at 95°C for 2 hour. This operation becomes necessary because of the negligible presence of water and high content of glycerol causing fatty acids to be present as mono- and diglyceride from 5% to 10% by weight of the total lipid content. As partial glyceride
5 esters tend to form W/O emulsion, the water addition and re-heating provides full saponification of the residual esterified fatty acid.

The mixture was transferred into a becker, then cooled to room temperature and 50% w/v sulfuric acid was added to the mixture which was stirred for 1 hour until the pH stabilized at about 3.

10 The acidulated oil phase formed a lower hydroglyceric layer and an upper fatty acid oil layer containing CLA, which was separated by decantating. Noteworthy, in industrial operation the separation could be carried out by centrifugation.

The CLA was washed with water and finally it was made
15 anhydrous with sodium sulphate and filtered, then it is stored in a dark bottle at 4 °C until time of use. Total yield about 770 g of an amber oil, whose GC-analysis is shown in Table 1.

TABLE 1

Fatty 20 Grape Seed Acid	Grape Seed	CLA from
	(Starting material)	(Final Product)
C14:0	0.11	0.13
C16:0	6.53	6.56

	C18:0	3.02	3.23
	C20:0	<u>0.19</u>	<u>0.20</u>
	total saturated	9.85	10.12
	C16:1	0.42	0.48
5	C18:1	16.42	17.15
	C18:1(t)	0.08	0.23
	C20:1	<u>0.59</u>	<u>0.60</u>
	total monounsaturated	17.51	18.46
	C18:2	72.11	1.76
10	C18:2-conjugated (CLA)	0.21	69.48
	C18:3	0.31	0.18
	C20:3	<u>0.01</u>	<u>0.00</u>
	total polyunsaturated	72.64	71.42

15 The composition of CLA appears to be a complex mixture, i.e. 9c,11t- and 8c,10t- octadecadienoic acids at 30,90 %, 11c,13t- 10t,12c- octadecadienoic acids at 32,05 %, 11t,13c- 8c,10c- 9c,11c- octadecadienoic acid at 1,55 %, 10c,12c- 11c,13c- 11t,13t , 9t,11t- 10t,12t- 8t,10t- octadecadienoic acids making the remaining part.

20 Example 2 – Synthesis of CLA-triglyceride with a high content of 9,11-octadecadienoates

A solution of 200 g of castor oil and 122 g of 4-dimethylaminopyridine (DMAP) in 900 ml acetonitrile was charged in a 3-neck round bottom flask (2 l) equipped with a magnetic stirrer, a
25 reflux condenser and a thermometer, then added dropwise over 2

hours with a solution of 190 g (1 mole) of toluene sulfonyl chloride in 300 ml acetonitrile. The mixture is left under stirring overnight at room temperature, then 400 ml water and 300 ml heptane were added, and the organic layer was separated and washed with 2x500 ml of dilute acetic acid, 3x1 l water, 1 l brine, and then it was dried and evaporated. The resulting oily product was dissolved in 900 ml DMSO, and 190 g DBU (diazabicycloundecene) were added. The reaction mixture was heated at 110°C and kept under stirring for 4 hours, then poured into water, extracted with hexane (500 ml), washed with dilute acetic acid, and water, then dried over sodium sulfate, to yield 172 g of a slightly yellow oil. Total yield about 156 g of an amber oil, whose GC-analysis is shown in Table 2.

TABLE 2

	Fatty	Chemically dehydrated
15	Acid	castor oil
	C16:0	1.45
	C18:0	<u>1.26</u>
	total saturated	2.71
	C16:1	0.21
20	C18:1	5.90
	C18:1 12-OH	3.59
	C20:1	<u>0.20</u>
	total monounsaturated	9.90
	C18:2	5.98

C18:2-conjugated (CLA)	80.72
C18:3	<u>0.69</u>
total polyunsaturated	89.90

The CLA fraction comprises about 90% of 9c,11t- and 9c,11c-
5 octadecadioic acids in a ratio of about 3:1, the remaining containing
minor amounts of the 9,12-isomers.

Example 3 and Preparative Example 1 - Evaluation of the peroxide
values of CLA from of grape seed oil vs safflower oil

The alkali isomerization of grape seed oil and safflower oil was
10 performed by a known procedure, i.e. the alkali isomerization in
diethylene glycol, to comparatively test the oxidation resistances of
the CLAs obtained from the aforementioned vegetal sources.

Thus, the isomerization method according to Nichols P.L. et al.
(JAOCS, 73, 247-252, 1951) was separately applied to 100 g of grape
15 seed oil and 100 of safflower oil, with only with minor modifications.

Briefly, 15 g of sodium hydroxide (NaOH), 100 of diethylene
glycol and 100 g of the oils were added into a 4-neck round bottom
flask (1000 ml) and the oils were heated at 210 °C under an inert
atmosphere for 3 hours.

20 The reaction mixtures were cooled to ambient conditions and
added with 2 parts of water and 50% w/v sulfuric acid until pH 3. The
mixtures were transferred into a separatory funnel and added with
heptane, then the upper phases were recovered, washed with water,
redried anhydrous with sodium sulphate and filtered, then the
25 heptane was evaporated under reduced pressure.

The products obtained from grape seed and safflower oil have a CLA content of about 65%, and 63%, respectively.

The two CLAs were tested by the AOM method for the determination of peroxide value. Briefly, 20 ml of each sample was placed in test tubes and thereby heated into an oil bath at 67°C. Fresh
5 air was then blown into the samples at the rate of 2.33 ml/s, then the time for the POV (peroxide value) of the oil to reach 100 was measured.

The grape seed CLA and the safflower CLA exhibit AOM values
10 of 4 hours and 2.5 hours, respectively. It is hereby confirmed that the alkali isomerization of grape seed oil may provide a CLA which is more resistant to oxidative conditions, thus having a higher shelf-life.

It should be understood that the specific forms of the invention herein illustrated and described are intended to be representative
15 only. Changes, including but not limited to those suggested in this specification, may be made in the illustrated embodiments without departing from the clear teachings of the disclosure.

CLAIMS

1. A process for the production of conjugated linoleic acid (CLA) characterized by reacting a linoleic acid containing vegetal oil with glycerol in presence of an alkali metal hydroxide.
- 5 2. Process according to claim 1, characterized by reacting a linoleic acid containing vegetal oil with glycerol in the presence of a suitable aqueous alkali metal hydroxide under inert atmosphere at a temperature ranging from 200 to 250°C.
3. Process according to claims 1 or 2, characterized by performing
10 the reaction for 1 to 5 hours then acidulating the reaction mixture comprising the crude CLA, recovering said CLA and optionally dehydrating, refining and deodorizing it.
4. Process according to claims 1 to 3, characterized in that the starting vegetal oil is a mixture of glycerides, fatty acids and
15 alkaline salts wherein the free linoleic acid content is at least 50% by weight of the total content fatty acids.
5. Process according to claims 1 to 4, characterized in that the vegetal oil is selected in the group consisting of grape seed oil, safflower oil, sunflower oil, soybean oil, distilled linoleic acid and
20 mixture thereof.
6. Process according to claims 1 to 5, characterized in that the alkali metal hydroxide is soda or potassium ash or mixture thereof.
7. Process according to claims 1 to 6, characterized in that the alkali
25 metal hydroxide to the vegetal oil ratio is from 1:1 to 1:3 molar equivalents.

8. Process according to claims 1 to 7, characterized in that glycerol to vegetal oil ratio is 1:2 to 2:1 w/w.
9. Process according to claims 1 to 8, characterized in that wherein the crude CLA is further diluted with water and reheated to achieve complete saponification.
10. CLA obtained by alkali isomerization of grape seed oil in solvents having a boiling point higher than 240°C.
11. A process for preparing a CLA-triglyceride having a high content of 9,11-CLA isomers from castor oil, which comprises bonding an appropriate leaving group in the 12-position of castor oil, adding mineral acids and cleaving said leaving group by a tertiary amine.
12. Process of claim 11 wherein the leaving group is tosyl or mesyl group.
13. Process of claim 11 wherein castor oil is reacted with tosyl or mesyl chloride in presence of a tertiary amine in an organic solvent.
14. Process of claim 11 wherein the tosylated or mesylated castor oil is diluted with mineral acid then reacted with a tertiary amine at high temperatures .